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Tissue-Specific Regulation of the *WT1* Locus

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The 11p13 Wilms' tumor locus consists of two coordinately regulated transcripts, *WT1* and *WIT-1*. These genes are highly expressed in the developing urogenital system, beginning with the urogenital ridge at day 10.5, the metanephric blastema at day 11.5, and during glomerular formation at day 13.5, becoming ultimately restricted to the podocytes. Stromal cells of the gonad also show abundant expression. *WT1* is expressed at lower levels in spleen, uterus, mesothelial linings of organs in the abdominal and thoracic cavities, and the ependymal layer of the ventral aspect of the spinal cord. *WIT-1* mRNA is about 10-fold less abundant than *WT1*, but appears to be expressed in the same tissue-restricted manner. Expression of the *WT1* protein is required for kidney development, although its physiological function remains to be determined. The function of *WIT-1* is similarly unknown but one intriguing possibility is that it is an antisense regulator of *WT1*. An understanding of events controlling spatial and temporal regulation of these genes will greatly improve our ability to study the role of *WT1* and *WIT-1* in urogenital development. We have found that while chimeric reporter constructs containing 0.6–2.5 kb of the 5′ region of the *WT1* gene direct transcription in many different cell lines, we were unable to detect expression in 13.5-day mouse embryos. However, a cosmid containing about 42 kb encompassing this region was able to direct the expression of abundant levels of mRNA from the appropriate transcription initiation sites in both stable transfectants of mouse Leydig cells (TM3) or in transgenic embryos. We are currently localizing the DNA elements required for this expression.

Key words: Wilms' tumor, kidney development, transgenic mice

INTRODUCTION

The *WT1* gene, originally identified on the basis of mutations and deletions in Wilms' tumors, has been shown to play a key role in urogenital development [1], consistent with its highly restricted pattern of spatial and temporal expression [2–6]. The *WT1* protein is a transcription factor, with four zinc fingers of the C_2H_2 type [7]. Various splice forms and mutations have been identified, producing proteins with differing DNA binding activities in vitro [8–9]. From a developmental point of view, little is known about the function of *WT1*. Potential downstream targets of *WT1* have been identified by transfection studies in cell culture [10–14], but their role in kidney development has not been rigorously tested. Pax-2 has been identified as a target gene in vivo, as its expression is absent in metanephric blastema of homozygously deleted *WT1* -/- embryos [1,15].

Studies from our laboratory and others have shown that the region immediately surrounding the *WT1* transcriptional start site cluster, can confer transcriptional control on heterologous reporter genes in transfected cells in culture [16–20]. However, the promoter was expressed in many cell lines, including several which do not express the endogenous *WT1* gene [16,19]. An enhancer activity was identified in the 3′ end of the *WT1* gene which is only active in K562 cells, and may play a role in the hematopoietic expression of *WT1* [19,21]. A silencer activity was also detected within the third intron of the *WT1* gene which suppressed expression of the *WT1* promoter in nonrenal cell lines [22].

We have shown that the minimal promoter region, as defined in cell culture studies, will not direct appropriate expression of *WT1* in transgenic mouse embryos. However, we were able to demonstrate robust *WT1* expression from a 42-kilobase (kb) cosmid containing the first three exons of *WT1* plus ~30 kb of 5′ sequence in both cultured cells and transgenic embryos. Efforts to localize enhancer elements within the 42-kb cosmid are currently in progress.

MATERIALS AND METHODS

The murine *WT1* basal promoter was cloned by screening a genomic lambda fix II library prepared from 129/SV DNA (Stratagene, La Jolla, CA) with a 170-bp probe derived by polymerase chain reaction (PCR) from the 3′ end of exon one of the murine Wilms' tumor (WT) gene representing nucleotides 750–920 [3]. Contiguous Hind

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III and Eco RI fragments were isolated and cloned into the luciferase reporter vector pGL2-Basic (Promega, Madison, WI). Transient transfection assays were performed with Lipofectamine (GIBCO/BRL, Gaithersburg, MD). Expression of *WT1*/luciferase constructs in transient transfections of TM3 cells were assayed according to the manufacturer's instructions using the Reporter Lysis Buffer protocol (Promega).

The pβGAL plasmid was constructed by blunting the 3.7-kb HindIII/BamHI fragment of pCH110 (Pharmacia) and inserting it into the EcoRV site of pBSII (Stratagene). The orientation was selected to be the same as the T7 promoter. The 2.5-kb PstI fragment of cosmid c2.2 (18) was cloned into the PstI site of pβGAL, and the 1.3-kb SacI/PstI and 0.6 HindIII/PstI fragments were blunted and inserted into the Sma I site of pβGAL. NotI/SalI digestion was used to purify the promoter/reporter fragment for transgenesis, and standard methods were used to produce FVB transgenic embryos [23]. Embryos were harvested at days 13.5–15.5 of development, fixed in 4% paraformaldehyde, cryosectioned (10 μm) and stained for β-galactosidase activity overnight at 30°C and counterstained with neutral red [23]. Trangenic mice were identified by Southern blot analysis of placental/yolk sac DNA.

The cosmid c2.2 was transfected into TM3 cells (ATCC) by calcium phosphate precipitation or Lipofectamine (GIBCO/BRL). Stable transfectants were selected in 400 mg/ml G418 (GIBCO/BRL). RNA was prepared by the guanidinium isothiocyanate/CsCl gradient method and 20 μg of total RNA was used for RNase protection using the 0.6 kb HindIII/PstI probe encompassing the *WT1* transcription start sites [18]. Transgenic embryos were prepared with cosmid c2.2 DNA linearized by the SalI site in the pWE15 vector. Total embryonic RNA was prepared by the RNAZOL B method (TelTest) and fifty μg was subjected to RNase protection as above.

BglII fragments of c2.2 were purified and inserted into the BamHI site of p1.3βGAL. Transfections and transgenic embryo analysis were performed as above.

RESULTS

A comparison of the human and murine *WT1* loci shows extensive sequence homology 5′ of the major *WT1* transcription initiation sites (Fig. 1A) [3]. Since minimal promoter activity for the human *WT1* gene has been established for fragments surrounding the transcription start site cluster, we determined whether this was also the case for the murine locus. The results (Fig. 1B) show that minimal bidirectional promoter activity can be assigned to a region in the murine locus homologous to the human counterpart.

To determine whether the minimal promoter defined in various cell lines would function in vivo in transgenic mouse embryos, β-galactosidase reporter constructs were injected into one-cell mouse embryos, transferred to pseudopregnant recipients, and harvested between days 13.5 and 15.5 of gestation, counting the day of injection as day 0.5. This time point was chosen on the basis of maximal expression of endogenous *WT1* within the kidney. There was no β-galactosidase staining detected in any tissue examined in either transgenic embryos or their nontransgenic littermates in 0.6, 1.3 or 2.5 βGAL embryos (data not shown). The results were obtained from 6 positive mice of 10 live births injected with the 2.5-kb promoter, 12 positive of 22 injected with the 1.3 kb promoter, and 3 positive of 7 injected with the 0.6 kb promoter. As similar constructs are reported to be active in various cell lines, yet are clearly not active in transgenic embryos, we concluded that in vivo expression of *WT1* requires additional regulatory sequences not required for transcription in cell cultures.

To identify those sequences, we performed transfection experiments with a cosmid, c2.2 (Fig. 2A), that contains the first three exons and introns of *WT1* as well as ~30 kb of 5′ sequence that includes most if not all of the divergently transcribed *WIT-1* gene [24]. The major transcriptional start sites of *WT1* and *WIT-1* are about 600 bp apart, but additional *WIT-1* transcripts, which have not been completely mapped but which initiate within the *WT1* gene [18], are likely to be contained within this cosmid. The cosmid vector, pWE15, includes an SV40 driven *neo* gene, which was used in selecting stable transformants in TM3, a mouse Leydig cell line which expresses abundant endogenous *WT1* mRNA. RNase protection using a probe that spans the transcriptional start sites for *WT1* showed that 3 of 4 stable transfectants expressed significant amounts of human *WT1* mRNA. Moreover, this transcription was initiated at the same start sites utilized in human fetal kidney (Fig. 2B).

Cosmid c2.2 was also injected into embryos that were harvested on day 13.5 and used to prepare total embryonic RNA. RNase protection was performed with the same probe as in Figure 2A. Two of three transgenic embryos show abundant expression of *WT1* RNA from the transgene (Fig. 3), while there are no protected products in a nontransgenic littermate. Since total embryonic RNA was used in these experiments it is not possible to draw any conclusion about transcription start site usage, as it may be different in different tissues or aberrantly expressed in inappropriate tissues. However, the fact that we observe abundant expression of the transgene in both TM3 and transgenic embryos makes it possible to address these issues.

The observation that short promoter constructs (<2.5 kb) gave us very low reporter activity in cells, while longer constructs (42 kb) directed the expression of significant amounts of mRNA, prompted us to begin identification of regions in the cosmid that were responsible for this expression. BglII fragments of the cosmid, ranging

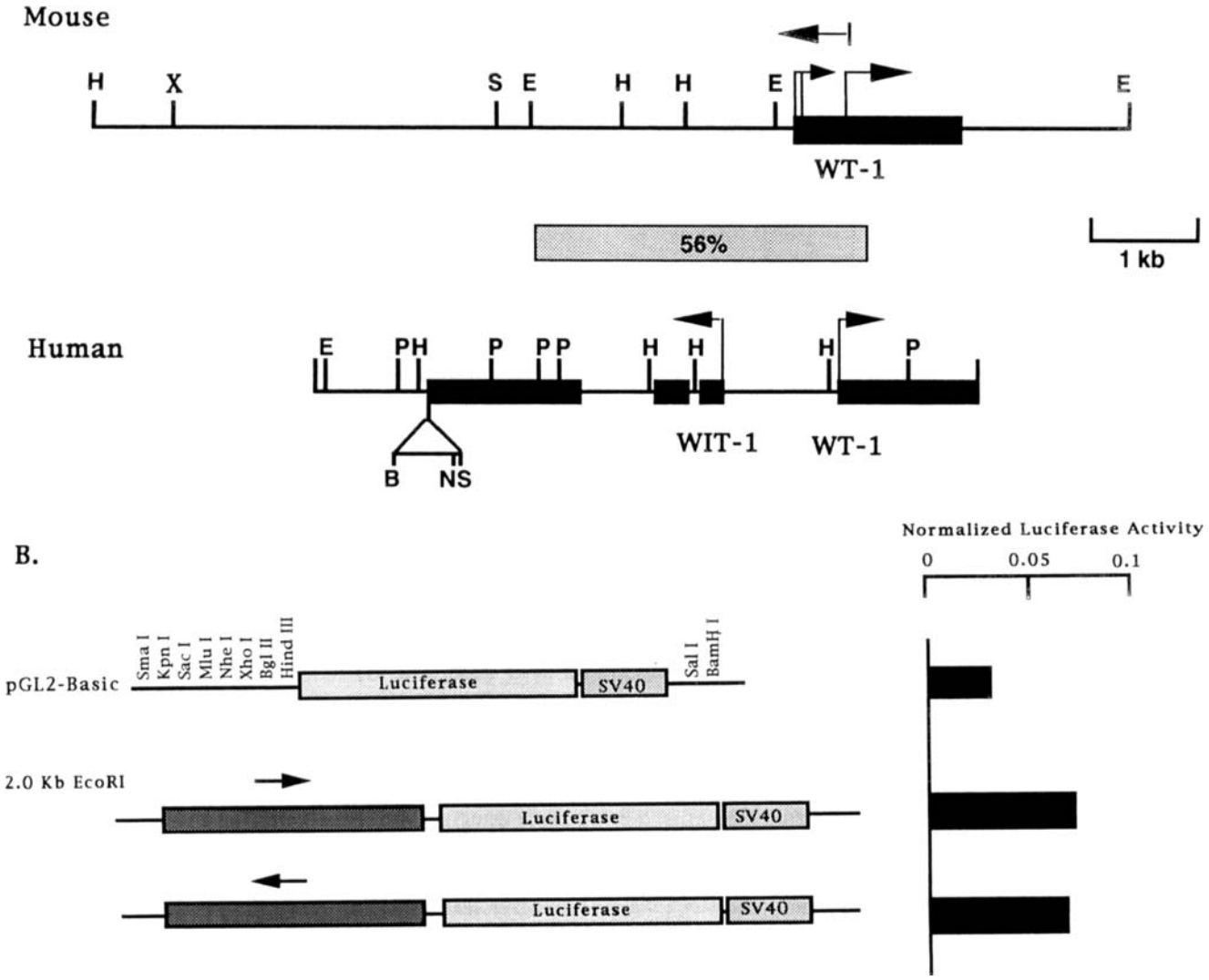

Fig. 1. **A:** Line drawing of mouse and human genomic fragments representing the Wilms' locus. The black boxes denote exons, the spaces between the boxes represent intron sequences. The transcriptional start sites are indicated by arrows. The arrow pointing to the left above the mouse sequence indicates antisense transcription of *WT1* as detected by RNase protection. The stippled bar indicates homology between mouse and human sequences over the marked region. A partial restriction map is represented as follows: E, EcoRI; P, PvuII; H HindIII; B BamHI. **B:** Transcriptional activity in transfected TM3 cells of a segment of mouse DNA homologous to the human Wilms' putative bidirectional promoter. The constructs used are shown at the left with arrows indicating orientation of the 2.0-kb EcoRI fragment (see A) This fragment was cloned for use in the forward direction by blunt-end ligation into the SmaI site of pGL2-Basic (Promega, Madison, WI). The reverse orientation was cloned into the XhoI/BglII sites of the pGL2-Basic vector. The data are presented as luciferase activity after normalization for cotransfected β-galactosidase activity.

in size from 500 bp to 8 kb, were inserted upstream of the 1.3-kb minimal promoter in the βGAL constructs used above (Fig. 4). These were initially transfected into TM3 cells, as they had given robust activity in earlier experiments. Figure 4 shows that while most constructs had activity similar to the 1.3-kb minimal promoter, the addition of fragment I from BGlII digestion of the cosmid enhanced transcriptional activity approximately fourfold. Therefore, we concluded that the 2-kb fragment I had some transcriptional enhancing properties. This same construct was injected into mouse embryos as before and harvested on embryonic day 13.5. There were 10 live embryos, of which 3 were positive by Southern blotting of placental/yolk sac DNA. However, sectioning and staining revealed no β-galactosidase activity in the kidney or in other tissues (data not shown).

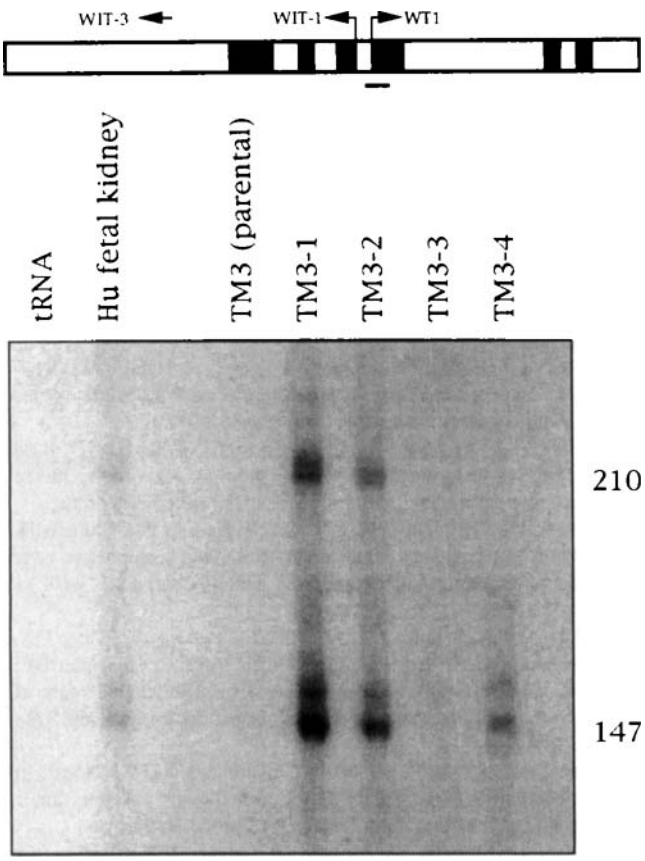

Fig. 2. **Above:** Map of cosmid and RNase protection probe. A schematic of the human DNA cosmid c2.2 is shown, with black boxes representing mapped exons. The major transcriptional start sites for *WT1* and *WIT-1* are shown, as well as the approximate location of a third transcript in this region, *WIT-3*. The line below the map indicates the position of the RNase protection probe used for *WT1* mRNA detection. **Below:** RNase protection of c2.2/TM3 stable transfectants. Twenty μg of total RNA was hybridized with the *WT1* probe, RNase digested and run on a denaturing gel. TM3-1 through TM3-4 are four different clonal lines. The sizes of protected products are indicated.

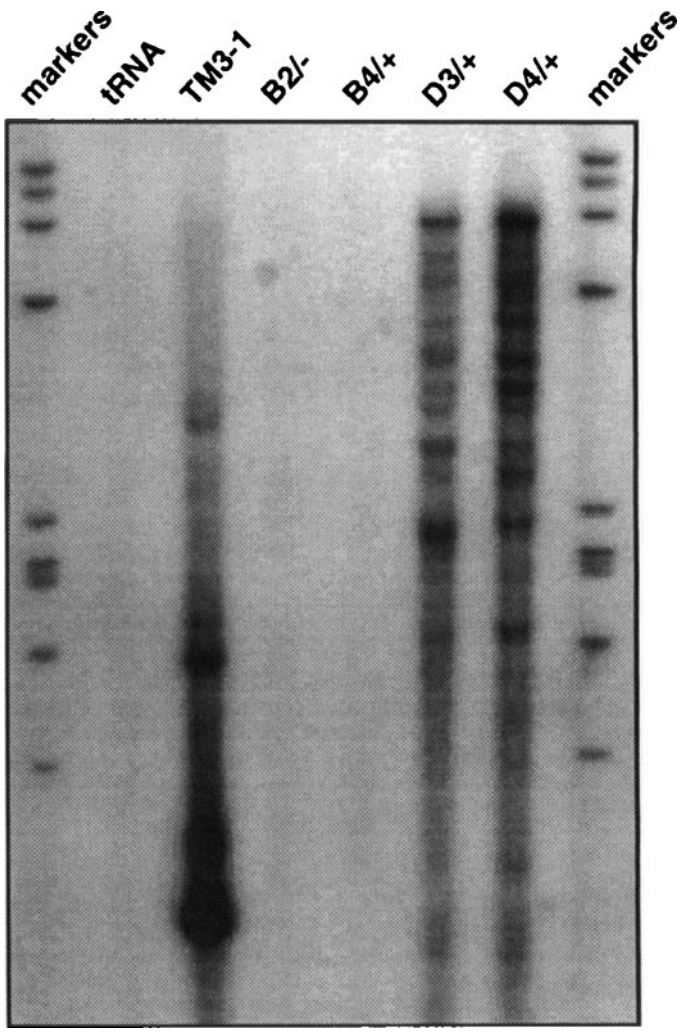

Fig. 3. Expression of c2.2 in transgenic embryos. Fifty μg of total embryonic RNA was hybridized with the same probe as in Figure 2. TM3-1 is the same clonal line as in Figure 2. Four embryos are presented here, indicated as positive or negative for the transgene. Hae III ϕx174 were kinased for markers.

DISCUSSION

Several reports have demonstrated transcriptional activity from 0.6 to 1.3 kb fragments of the promoter region of *WT1* in transfected cultured cells. We have referred to this activity as basal promoter activity [18], while others have referred to it as weak or promiscuous [16,19]. This level of activity was sufficient to demonstrate negative autoregulation by *WT1* overexpression in two studies [17,20]. Our conclusions from the experiments presented here are that this basal promoter region, while active in cell lines, is not active in transgenic embryos. We have chosen for analysis embryonic day 13.5, at which time endogenous *WT1* is expressed at high levels in a discrete pattern in the kidney and gonads, as well as at lower levels of expression in other tissues, such as spleen and mesothelium [6]. The results suggest that the identification of sequences required for the appropriate temporal and spatial regulation of the *WT1* locus in vivo will require the use of transgenic mouse embryos, in which all of the appropriate developmental signals are present.

Our efforts to identify these additional regulatory elements were initiated by using a 42-kb cosmid that contains the 5′ end of the *WT1* gene (exons 1 to 3) and ~30-kb of 5′ upstream sequence which includes most, if not all of the *WIT1* gene. Using this cosmid, we have demonstrated robust transgene expression both in the TM3 cell line and in transgenic mouse embryos. Moreover, as demonstrated by RNase protection, the correct transcriptional start sites are utilized in TM3 cells. To our knowledge this is the first demonstration of appropriate transcription start site usage in a transfected *WT1* promoter assay. It should be noted that this cosmid contains a *WT1* gene truncated within the third intron and therefore is missing

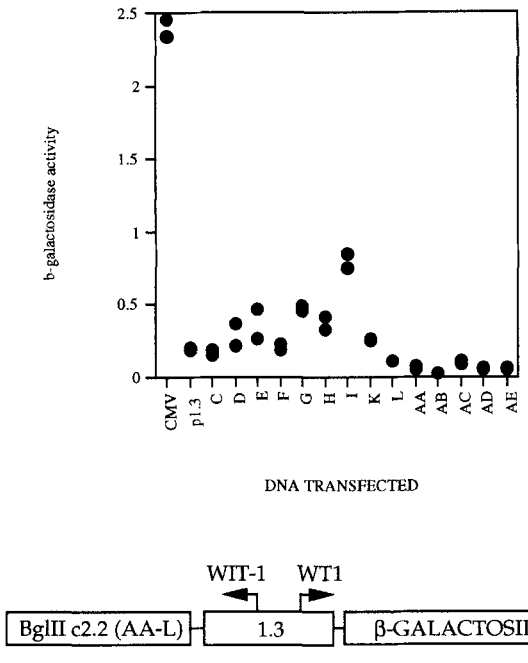

Fig. 4. Expression of β-galactosidase activity in TM3. Various BglII fragments of c2.2, ranging in size from 8 to 0.5 kb, were subcloned into the p1.3βGAL vector as shown. These constructs were transfected transiently into TM3 cells using Lipofectamine and assayed for β-galactosidase activity. CMVβGAL was used as a positive control for transfection. Dots represent duplicate samples.

its polyadenylation signal. We have not yet ascertained whether the transcripts being synthesized lack a polyA tail or are polyadenylated using a cryptic signal within the cosmid.

To identify the DNA elements in the cosmid responsible for transcriptional activity, we have made a series of reporter constructs consisting of the minimal 1.3-kb promoter fragment plus additional 0.5- to 8-kb fragments of the cosmid derived from sequences both 5′ and 3′ of the *WT1* transcriptional start sites. Having tested ~85% of the cosmid insert, no strong enhancer elements have been unequivocally identified. It is possible that the *WT1* enhancer(s) we seek lies in the remaining 15% of DNA still to be tested. Alternatively, the element may have been destroyed by the restriction digests used to dissect the cosmid. By digesting with a different restriction enzyme, we could perhaps keep intact such an enhancer. However, before pursuing these experiments, an alternative approach such as identifying DNase I hypersensitive sites within the transfected cosmid or endogenous murine *WT1* gene should provide important information in identifying the temporal and spatial elements regulating this locus.

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COMMENTARY

Hewitt et al. remind us of the possibility that a second gene, *WIT-1*, localized at chromosome 11p13 and very close to *WT1*, may play a role in urogenital development and Wilms' tumorigenesis. Although the function of *WIT-1* remains unknown, these investigators demonstrate that *WIT-1* mRNA expression which is about 10-fold less abundant than *WT1* expression, appears to be expressed in the same tissues and during the same stages of organ development as is *WT1*. The authors speculate that *WIT-1* might function as an antisense regulator of *WT1*.